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Constraints on Maternal Ability to Adjust Sex Ratios in Mammals

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“Explanations exist: they have existed for all times, for there is always an easy solution to every problem - neat, plausible and wrong.”

H.L. Mencklen, 1917.



Statements by the author

Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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The research associated with this thesis abides by the *Australian code of practice for the care and use of animals for scientific purposes* (2004) and the rulings of the Animal Ethics Committee of the University. The research presented in this thesis was carried out under University of Tasmania Ethics Approvals A12366 and A13748.

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Date: 24 March 2016

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Chapter 1 has been published in *Journal of Zoology*:

Edwards, A.M., Cameron, E.Z. and Wapstra, E. (2016) Are there constraints on maternal ability to adjust sex ratios in mammals. *Journal of Zoology*. 299, 1-9.

Candidate was the primary author and contributed approximately 60% to the planning, execution and preparation of the research project and subsequent paper.

Authors 2 and 3 contributed ideas and assisted with writing the manuscript.

Chapter 2 has been published in *Proceedings of the Royal Society Open Science*:

Edwards, A.M., Cameron, E.Z., Pereira, J.C., Wapstra, E., Ferguson-Smith, M.A., Horton, S.R. and Thomasson, K. (2016). Gestational experience alters sex allocation in the subsequent generation. *Royal Society Open Science*. In Press.

Candidate was the primary author and contributed approximately 50% to the planning, execution and preparation of the research project and subsequent paper.

Author 2 assisted with the conception and contribution of ideas, experimental design, experimental procedures and writing the manuscript. Author 4 designed and assisted with laboratory techniques. Author 3 assisted with experimental design and writing the manuscript. Author 5 designed and assisted with laboratory techniques and writing the manuscript. Authors 6 and 7 assisted with experimental procedures.

Chapter 3 is under revision with *Behavioural Ecology*:

Edwards, A.M, Cameron, E.Z., Wapstra, E. and McEvoy, J. (2016) Environmental mismatch: experimental evidence that maternal effects constrain sex allocation in the next generation. *Behavioural Ecology*. In Review.

Candidate was the primary author and contributed approximately 55% to the planning, execution and preparation of the research project and subsequent paper.

Author 2 assisted with the conception and contribution of ideas, experimental design, experimental procedures and writing the manuscript. Author 3 assisted with experimental design and writing the manuscript. Author 8 assisted with animal husbandry and writing the manuscript.

Chapter 4 has been published in *Trends in Ecology and Evolution*:

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Candidate was the primary author and contributed approximately 60% to the planning, execution and preparation of the research and subsequent paper. Candidate undertook the data collection from the literature. Candidate and Author 2 equally contributed to the conception, ideas and writing of the manuscript.

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Author 2 assisted with the conception of the idea, experimental design, experimental techniques and writing the manuscript. Authors 4 and 5 assisted with designed and assisted with the laboratory techniques and writing the manuscript.

Chapter 6 has been published in *Reproduction, Fertility and Development*:

Edwards, A.M. and Cameron, E.Z. (2016) Cryptic male choice: experimental evidence of sperm sex ratio and seminal fluid adjustment in relation to coital rate. *Reproduction, Fertility and Development. In Press.*

Candidate was the primary author and contributed approximately 70% to the planning, execution and preparation of the research project and subsequent paper.

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Abstract

Sex allocation theory predicts that mothers would benefit from sex-biased differential investment into offspring in relation to their current local condition when it maximizes their lifetime reproductive return. In mammals, however, the extent of the sex bias at birth is often unpredictable, suggesting that mothers may be constrained in their ability to adjust sex ratios. None of the current hypothesized mechanisms of peri-conceptual mammalian maternal sex allocation fully explain the amount of variation observed, and as such I suggested three possible physiological constraints on maternal sex allocation. Firstly, mothers may be constrained by variation in physiological traits, particularly mediated during their own early development through *in utero* effects, such as testosterone levels and responsiveness to stress. I tested the effects of physiological changes caused by down-regulated stress during *in utero* development, and showed significant physiological changes in females, as a result of mismatching pre- and post-natal environments, that skewed sex ratios in the next generation. However, artificially lowering the stress of these females at conception will cause the sex ratio to return to parity, as the pre- and post-natal environments match again. Secondly, their physiology may be influenced more proximally, by not only their current condition or ability to invest, but by clinically asymptomatic disease and parasitic infection, particularly manipulative parasites. Lastly, paternal influences such as sperm sex ratios and seminal plasma constituents have been largely overlooked but may influence and constrain maternal ability to adjust sex ratios. I showed evidence of variations in sperm sex ratios, both in the literature and through observational studies where we would expect parity as a result of meiosis during sperm production. I also presented the first evidence of paternal sex allocation, through changes in sperm sex ratios and seminal plasma constituents in relation to coital rate, as a proxy of male attractiveness. The possibility of complementary or antagonistic interactions between maternal and paternal sex allocation should now be accounted for in future research. Overall, my thesis has provided explanations into previously unexplained variation in sex allocation research, and may assist with improvements to conservation breeding and livestock industries, as well as human health developments.

Notes on Text

This thesis consists of published papers and submitted manuscripts, therefore each chapter is set out largely in the style of the journal to which it has been submitted; those chapters that have been published are inserted as copy edited PDFs, where available. Consequently, there is some repetition, particularly in the introduction and methods sections, and there are also stylistic differences between chapters.

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Chapter 1: Part I

Theoretical Background

Edwards, A.M., Cameron, E.Z. and Wapstra, E. (2016). Are there physiological constraints on maternal ability to adjust sex ratios in mammals? *Journal of Zoology*. **299**: 1-9.



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Edwards, A.M., Cameron, E.Z. and Wapstra, E., (2016), Are there constraints on maternal ability to adjust sex ratios in mammals, *Journal of zoology*, 299, 1-9.

Chapter 1: Part II

Thesis Outline



Section I comprises Chapters 2-3 and investigates constraints resulting from lifelong and inter-generational modifiers of maternal physiology.

Chapter 2 presents evidence that suggests that an artificially adjusted environment during late gestation is capable of constraining a mother's ability to adjust the sex ratio of her offspring through maternal effects, an example of lifelong and inter-generational modifiers of maternal physiology. This chapter has been published in *The Royal Society Open Science*.

In **Chapter 3**, I investigated whether simulating the same artificial environments (Ch2) pre- and post-natally can return the sex ratio to parity. This chapter confirmed that maternal effects constrain a female's ability to respond to environmental factors. This chapter is under review with *Behavioural Ecology*.

Section II comprises Chapters 4-6 and investigates the constraints imposed by fathers.

In **Chapter 4**, I present literature-based evidence of the possibility of paternal sex allocation and adaptive control by fathers. I present some possible mechanisms and discuss the outcomes of the interactions with maternal sex allocation. This chapter is a literature review that was published as an Opinion Review in *Trends in Ecology and Evolution*.

Chapter 5 presents data that supports our arguments from Chapter 4, and was published as an original research paper in the *Journal of Zoology*. I show that even laboratory mice raised under standardised conditions, present with sperm sex ratios that differ from the expected 50:50 ratio. I also show significant individual variation between males who were raised in the same environment and provide further support for the possibility of adaptive control by fathers.

Chapter 6 empirically tests the effects of male coital rate, as a proxy for attractiveness, on sperm sex ratios and ejaculate components, by manipulating male access to female mates in laboratory mice. This chapter provides evidence in support of the mechanism and supports our novel idea that ejaculate components may be a form of male cryptic choice, further constraining maternal sex allocation. This chapter has been published in *Reproduction, Fertility and Development*.

Finally in **Chapter 7**, a general discussion evaluates the findings of the study, and makes recommendations for future research into parental sex allocation.

Section I:

Lifelong
and Inter-
generational
Modifiers
of Maternal
Physiology



Chapter 2

Gestational experience alters sex allocation in the subsequent generation

Edwards, A.M., Cameron, E.Z., Pereira, J.C., Wapstra, E., Ferguson-Smith, M.A., Horton, S.R. and Thomasson, K. (2016). Gestational experience alters sex allocation in the subsequent generation. *Open Science*. In Press.



Abstract

Empirical tests of adaptive maternal sex allocation hypotheses have presented inconsistent results in mammals. The possibility that mothers are constrained in their ability to adjust sex ratios could explain some of the remaining variation. Maternal effects, the influence of the maternal phenotype or genotype on her developing offspring, may constrain sex allocation through physiological changes in response to the gestational environment. We tested if maternal effects constrain future parental sex allocation through a lowered gestational stress environment in laboratory mice. Females that experienced lowered stress as embryos *in utero* gave birth to female biased litters as adults, with no change to litter size. Changes in offspring sex ratio was linked to peri-conceptual glucose, as those females that had increasing blood glucose peri-conceptionally gave birth to litters with a higher male to female sex ratio. There was, however, no effect of the lowered prenatal stress for developing male embryos and their sperm sex ratio when adult. We discuss the implications of maternal effects and maternal stress environment on the life-long physiology of the offspring, particularly as a constraint on later maternal sex allocation.

Key words

Sex allocation, maternal, paternal, fluorescent in-situ hybridisation, sex ratio

Introduction

Adaptive sex allocation hypotheses predict variation in the sex ratio of offspring where sex-specific fitness returns vary with local conditions and/or parental ability to invest [1-4]. Such hypotheses are logically appealing, and have resulted in numerous empirical tests, including in mammals [reviewed in 5, 6, 7]. Initial reviews in mammals suggested little consistency in support for adaptive hypotheses, but methodological inconsistencies between studies explain some of the variation [5, 7]. Nonetheless, the vast majority of variation remains unexplained, both between and within species in empirical studies occurs, especially in mammals [8]. The unpredictability of effect sizes suggests that parents may be physiologically constrained in their ability to skew the sex of their offspring [9, 10].

An increasing understanding of the underlying physiological mechanisms for maternal sex allocation suggests factors that might constrain maternal ability to skew sex ratios [10]. Lifelong and inter-generational modifiers of maternal physiology may constrain an individual's ability to respond to the current local conditions [10-12], particularly through maternal effects, the causal influence of the maternal phenotype or genotype on developing offspring [13-15]. Several factors have been linked to sex ratio skews through their physiological actions, including circulating glucose [5], testosterone [16-18] and stress hormones [19]. Each of these factors is influenced by the local conditions a mother experiences, and can directly affect the developing foetus. Thus, the environment experienced *in utero* can alter physiological pathways, thereby changing the individual's response to the environment as adults [20]. Such maternal effects may result in parents that are physiologically constrained in their ability to alter sex ratios in response to current conditions.

Stress responses provide a link between the proposed mechanisms of sex ratio adjustment [19, 21], and can have profound physiological impacts on developing offspring as a maternal effect [22]. Stressors experienced by the mother are mediated through internal hormone fluctuations; stressors stimulate the release of corticotropin-releasing hormone from the hypothalamus, which in turn stimulates the

release of adrenocorticotrophic hormone from the pituitary gland, resulting in the release of glucocorticoids [GCs; 23]. GCs then bind to receptors, which allow the body to return to homeostasis through acute stress events [23-25]. Foetuses are extremely sensitive to GCs [26, 27], and so protective enzymes (e.g. 11 beta-hydroxysteroid dehydrogenase type 2) in the placenta metabolise roughly eighty per cent of naturally occurring GCs, thereby buffering the foetus from high levels of GCs [28, 29]. However, the remaining proportion can cross the placenta, and thereby influence offspring development [30]. These changes can be either deleterious or advantageous to the offspring [e.g. 31, 32], and can last a lifetime [31], potentially even persisting across generations [33, 34]. Offspring fitness may be increased, for example by matching poor-quality mothers with reduced offspring demand [35], and offspring traits that increase survival [32]. However, changes that create a mismatch with the local environment are likely to result in offspring relatively less suited for the current environment, thus decreasing their fitness [36, 37].

The physiological effects of maternal gestational stress on developing offspring include changes in the hypothalamic-pituitary-adrenal (HPA) axis function, immunity, glucose and insulin tolerance and regulation, body condition and adult reproductive behaviour, and function in the offspring [38-40]. Stress likely influences maternal sex allocation, through increased susceptibility of male offspring to adverse conditions during late gestation [41], and more subtly through physiological changes persisting into adulthood. Changes to the HPA axis (and thereby sensitivity to stress) as a result of maternal effects during late gestation could influence offspring sex ratios and survival once that offspring itself reaches breeding age. Furthermore, such changes may influence maternal sex allocation through interactions with free glucose [5], since hepatic gluconeogenesis results from increased cortisol [42], and gestational stress can alter glucose levels and insulin tolerance lifelong [43, 44]. Increases in peri-conceptual glucose increase the proportion of male offspring [5, 45], due to interactions between free glucose and X-linked proteins and metabolic pathways [46], where female conceptus development is compromised under high glucose conditions [45, 47] but enhanced under low glucose conditions. GCs also inhibit the secretion of reproductive hormones, including testosterone, also linked to sex ratio skews in mammals [48]. High levels

of maternal testosterone have been linked to an increasing proportion of male offspring [49, 50], hypothetically altering the receptivity of the egg to either X- or Y-chromosome-bearing spermatozoa in relation to follicular testosterone [17]. Hormonal differences between adult males have also been linked to variation in the X to Y ratio in sperm [reviewed in 9] potentially also influencing paternal sex allocation. Therefore, maternal stress levels can influence offspring development during gestation in ways that could alter sex allocation when they reproduce, irrespective of current local conditions.

Here, we test if down-regulated stress during late gestation in laboratory mice impacts 1) the physical development and reproductive success of offspring, and 2) their sex allocation, in terms of sperm sex ratios in adult males and birth sex ratios in females. We predict that offspring born to treated mothers will have an increased number of glucocorticoid receptors [51], and therefore increased susceptibility to stress [26]. Female offspring may then experience increases in offspring sex ratios as a result of increased gluconeogenesis [5], however we don't predict that these changes should influence male sperm sex ratios.

Methods

We used BALB/c mice bred and housed at the University of Tasmania, Australia. They were kept under 12hr L:D photoperiod in a temperature and humidity controlled room, and provided with mouse chow (Barastoc® irradiated food) and filtered water *ad libitum*.

Generating Focal Females & Males

The experimental design is outlined in Figure 1. Forty nulliparous dams were housed in groups of up to 5 until 7 weeks of age when they were separated into pairs. One male was introduced to each cage, and each morning the dams were checked for the presence of a copulatory plug. Those dams that had a copulatory plug were removed from the cage and placed into group cages. The dams that did not have a copulatory plug were left with a male until a plug was observed.

We used dexamethasone to reduce stress in these pregnant dams in late gestation. Dexamethasone is a synthetic GC that simulates an artificial low stress environment [52, 53], and is used during late gestation in humans to reduce the risk of respiratory distress syndrome in premature babies [22]. Foetal effects from the simulated low stress environment are expected to be exaggerated because dexamethasone is not metabolised by the placenta [54]. Thus, there are fewer maternal GCs crossing the placenta as a result of dexamethasone interacting with the mother's body, as well as free dexamethasone entering the foetus and blocking its naturally occurring GCs. Combined, these effects result in perceived low stress levels for offspring.

At day 16 after the presence of a copulatory plug, $1.0 \mu\text{g ml}^{-1}$ of dexamethasone [as used by 52] was added to the drinking water of 22 dams, and this was replaced with fresh water after 3 days. Although this method results in variable dosages, it eliminates any increase in GCs from the stress of handling and injections [53], which potentially could negate the treatment [52]. Water-soluble dexamethasone is provided in a complex with 2-hydroxypropyl- β -cyclodextrin. Therefore, we had 10 dams whose water was treated with $14.4 \mu\text{g ml}^{-1}$ 2-hydroxypropyl- β -cyclodextrin as a vehicle control, to equally match the amount of vehicle that was required to deliver $1.0 \mu\text{g ml}^{-1}$ of dexamethasone. The water of 8 dams was left untreated, as the negative control.

As close as possible to birth and at most within 10 hrs, the pups were counted to record litter size in case of infanticide. These pups are considered to be the focal animals; the sperm sex ratios and offspring sex ratios produced by them are a means of determining the influence that maternal stress had. At 21 days after birth, the focal pups were sexed via visual examination of the anogenital distance and separated into single sex group cages. To avoid pseudoreplication, only one focal female and one focal male from each litter were kept as the focal animals. At 7 weeks of age the focal pups were considered adult, and body measurements (Table 1) were taken.

Breeding of Focal Females

Focal females were housed in pairs with an unrelated male until a copulatory plug was noted, after which females were weighed and blood glucose tested. Three days later the blood glucose test was repeated, to calculate the change in periconceptual blood glucose level. Focal females were allowed to birth naturally and pups were again sexed using anogenital distance. Seven focal females did not conceive, and a further two committed infanticide prior to offspring sexing and were removed from the analysis. The final sample size was 31 (Figure 1). The sex ratio of the resultant litter was recorded.

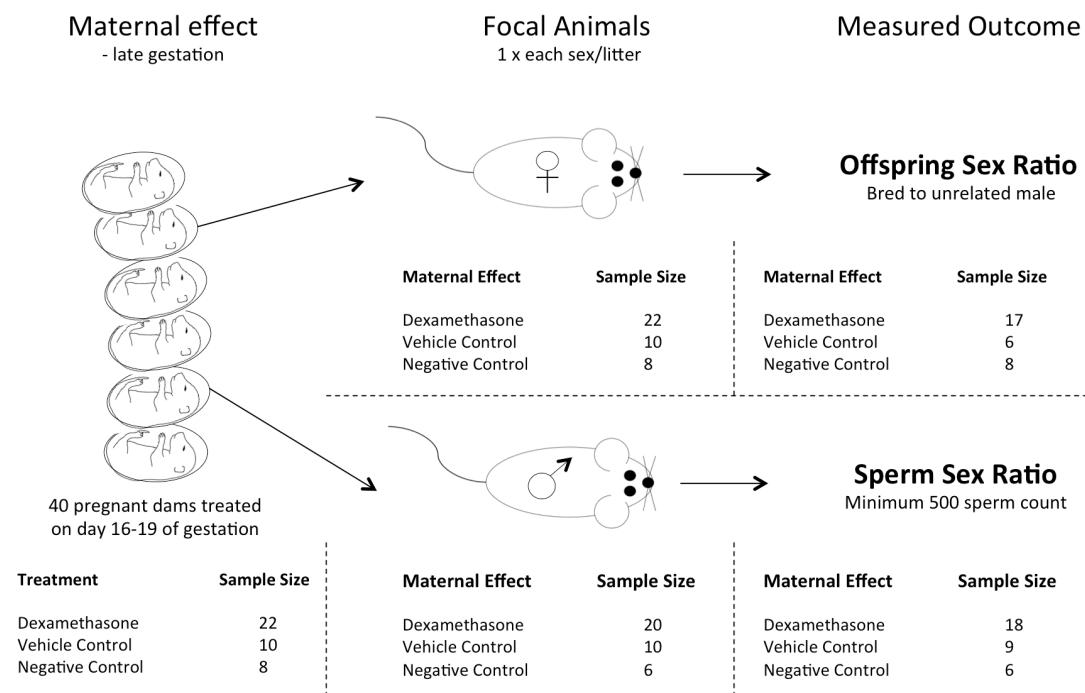


Figure 1. Diagram of the experimental design. The sample sizes at each stage of the experiment are listed based on treatment.

Sperm Collection from Focal Males

Focal males were sacrificed via cervical dislocation at between 67 and 74 days of age. The left epididymis and vas deferens were dissected into 0.5ml cryopreservation media (18% raffinose + 3% skim milk). The semen was squeezed from the vas

deferens using tweezers and allowed to swim out of the epididymis through lateral incisions. The resultant sperm suspensions were stored in straws and cryopreserved in liquid nitrogen.

Table 1. Variables measured from BALB/c mice, used in a mating trial to determine whether maternal effects (in utero treatment with dexamethasone) have the ability to constrain sex allocation in laboratory mice. Physical body measurements were taken at maturity (7 weeks of age).

Variable	Description
Body Condition	Calculated from the residuals of an ordinary least squares linear regression of body mass and pes length [60]. Pes length is measured using digital callipers.
Anogenital Distance	Calculated as the distance between the anus and the genital opening. Measuring using digital callipers.
Digit Ratio	Digit ratio was calculated as the ratio of second to fourth digit on the hind right foot. Digit length is measured using digital callipers from the tip of the toe to the base of the footpad. Observers were blind to the treatment of the animal.
Blood Glucose	Blood glucose was measured using an Accu-Chek Performa Nano glucometer, from blood collected via tail tipping.

Fluorescence in-situ hybridization on Sperm

The full methods are described in Edwards *et al.* [55]. Briefly, the sperm samples were washed and fixed to glass slides, decondensed and treated with pepsin prior to denaturation in 70% formamide. The X-chromosome probes were labelled with Cy3, and Y-chromosome probes with biotin. Denatured probes were added to the slides and hybridizations were performed in a warm, moist chamber for 24-48 hours. Slides were washed and detection of the Y-chromosome probe was performed using avidin-

fluorescein isothiocyanate (FITC), prior to counter staining the sperm heads with 4'6-diamidion-2-phenylindole ml^{-1} (DAPI) and mounting using an anti-fade solution (Vectashield, Vecta Laboratories, CA). Sperm were observed using a Leica DMRXA fluorescence microscope, with Cy3, FITC and DAPI specific filters. A minimum of 500 spermatozoa were counted per individual, from images collected using Leica QFISH with a cooled CCD camera through $\times 40$ or $\times 63$ oil-immersion objectives.

Of the 40 initial litters, four did not produce any males, three sperm samples were destroyed during transportation, and one sample failed to hybridise sufficiently for analysis, resulting in 33 focal males (Figure 1).

Statistics

All analyses were performed in R version 3.2.2 [56].

Focal Female Offspring Sex Ratio Analysis

Binomial generalised linear models with an intercept of 1 were run to determine whether the treatment group, or either control group presented with sex ratios different to the predicted 50:50 ratios. These results are presented as 95% confidence intervals on the estimate.

A generalised linear model with binomial error was run to determine whether periconceptual change in glucose, treatment or body condition influenced the sex ratio of offspring. This model also included an interaction effect between periconceptual glucose and treatment. While a multivariate analysis of variance (MANOVA) was run to determine whether the treatment had any effect on the physical body measurement of focal animals. An analysis of variance (ANOVA) was also run to determine whether litter size varied with treatment.

Focal Male Sperm Sex Ratio Analysis

A full generalised linear model with binomial error was run to determine whether treatment or body condition influence the sex ratio of sperm. While a MANOVA was

run to determine whether the treatment had any effect on the physical body measurement of focal animals.

Results

Litter Sex Ratios

The treatment group produced sex ratios that were significantly lower than the predicted 50:50 ratio (GLM: -0.943, -0.161; Figure 2), whereas neither control group differed from parity (GLM negative control: -0.798, 0.274; GLM vehicle control: -0.922, 0.738).

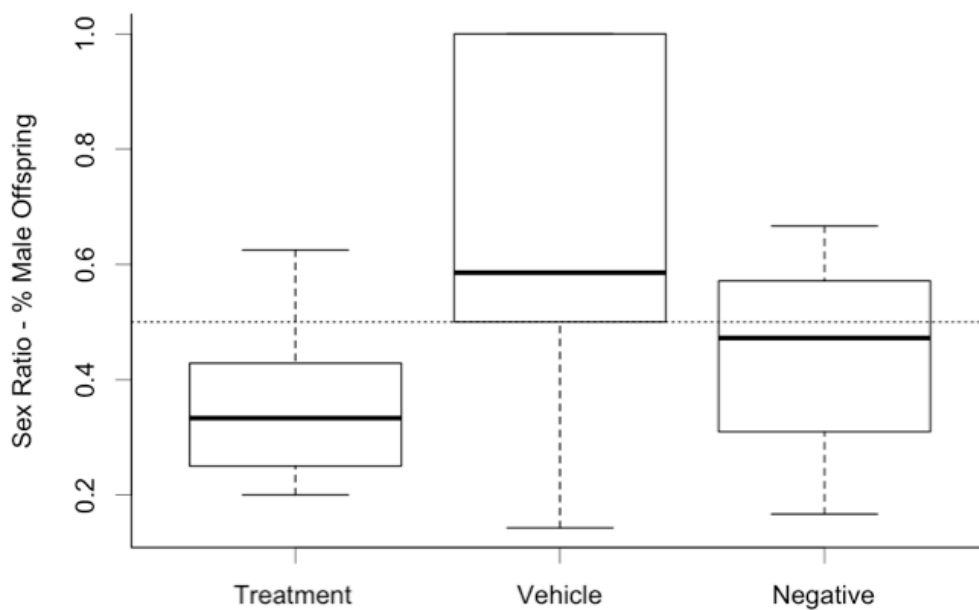


Figure 2. Female mice that receive dexamethasone treatment in utero produce litters with sex ratios that are lower than the expected 50:50 ratio, but females who received the vehicle or untreated water did not. The dotted line indicates the expected 50:50 ratio.

The sex ratio of offspring was significantly influenced by periconceptual change in glucose ($\text{Pr}(>\text{Chi})_{1,29} = 0.033$; Figure 3), but not by treatment ($\text{Pr}(>\text{Chi})_{2,27} = 0.676$) or body condition ($\text{Pr}(>\text{Chi})_{1,26} = 0.915$). There was also no interaction effect between the change in periconceptual glucose and treatment ($\text{Pr}(>\text{Chi})_{2,24} = 0.554$). The treatment did not result in a change in litter size ($F_{2,28} = 3.174$, $P = 0.057$), however there was a slight trend for the vehicle control group to have smaller litters. The treatment also did not influence the physical and physiological body measurements of the focal animals ($F_{10,48} = 0.955$, $P = 0.493$).

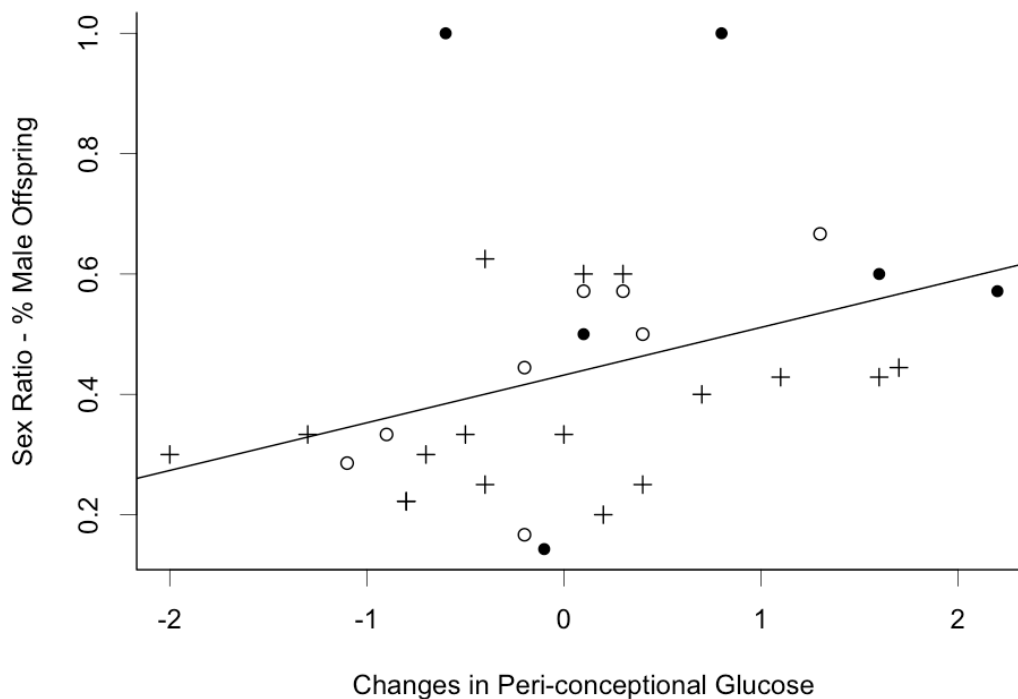


Figure 3. The linear relationship between sex ratio (as percentage of male offspring) and peri-conceptual blood glucose changes from day 0 to day 3 after confirmed copulation in laboratory mice. Crosses represent the sex ratios of females who received dexamethasone treatment during late development (in utero). Filled circles represent females who received the vehicle control and open circles represent females that did not receive any treatments.

Sperm Sex Ratios

The sperm sex ratio was not significantly influenced by treatment ($\Pr(>\text{Chi})_{2,30} = 0.192$) or body condition ($\Pr(>\text{Chi})_{1,29} = 0.488$). There was also no effect of treatment on any physical or physiological body measurement of the focal males ($F_{8,56} = 0.975$, $P = 0.477$).

Discussion

Maternal effects altered focal female sex ratios, but not the X- and Y-chromosome ratio in focal male sperm. Females that received the dexamethasone treatment during late-gestational development gave birth to litters with sex ratios lower than the predicted 50:50 ratio, with no change to litter size. However, increases in blood glucose were more strongly associated with an increase in male offspring than treatment *per se*, suggesting that environmental interactions with glucose metabolism may be more influential than maternal effects.

The developmental impacts of late gestational maternal stress manipulation influence stress responses and glucose metabolism in later life [22]. Embryonic female guinea pigs exposed to dexamethasone *in utero* have increases in glucocorticoid receptor and mineralocorticoid receptor mRNA in all regions of their hippocampus, and altered GC levels which are lower in the luteal phase but higher during oestrous [22]. However, increases in cortisol are associated with hepatic gluconeogenesis [42], and an overall increase in glucose [57]. Therefore, the lowering of cortisol levels during the luteal phase and the observed increase in female offspring might be better explained through the glucose hypothesis [5], through associated low levels of gluconeogenesis, and therefore, an overall decrease in free glucose.

In this study, the focal females that had an increase in blood glucose levels over the time of conception and early gestation give birth to more sons. This provides further evidence in support of the glucose hypothesis [5], where early blastocyst females survive better in low glucose environments, and males in high glucose environments [45]. Change in blood glucose levels significantly influence sex ratios while

treatment only did so indirectly through an interaction with glucose levels, probably due to the delivery method, since drinking water results in variable dosages [52]. However, as dexamethasone was used to simulate low stress, variable dosage was preferable to negating the treatment from injection-induced stress [52, 53].

The possibility of maternal effects constraining a father's sperm production has not been previously investigated. No significant shift in sperm sex ratios of the focal males is unsurprising, as we do not anticipate that stress or changes to HPA axis functioning should affect sperm production. Unlike mothers, mammalian fathers do not require large energetic investment in the production of gametes [58], or even in the offspring themselves [58], and therefore, changes to stress pathways are unlikely to influence paternal sex allocation. However, research into paternal sex allocation and the possibility of adaptive control by fathers is limited [9, but see 59, 60, 61], and it is unknown under what circumstances paternal sex allocation could occur [9, 55], although James [62] has suggested a role for pre-mating androgens in fathers.

There were no changes to the physical appearance of either sex offspring, even though previous studies on gestational dexamethasone have shown variation in physical characteristics [reviewed in 63]. Many of the studies that have presented offspring with physical changes have used much larger intravenous or subcutaneous dosages, and even multiple dosages, which leads to greatly exaggerated effects [63]. In comparison our dosage was high enough to have physiological effects on subsequent sex ratios (suggesting changes to underlying physiology) but not enough to have deleterious effects on offspring morphological development. In addition, we found no evidence that testosterone was linked to sex allocation. We measured both the digit ratio and the anogenital distance of the mice, which are indicative of the female's prenatal androgen exposure [64], but neither of these were correlated with sex ratio. There is contention regarding the use of digit ratios as androgen exposure indicators [65], and, therefore, although our data shows no support for a role of testosterone, we cannot rule out a role for testosterone influencing sex ratios.

We have shown that the gestational environment results in female offspring whose physiology is altered in a way that affects their reproductive functioning as an adult,

which could influence the success of management and captive breeding programs. Changes to female physiological pathways due to maternal effects can constrain maternal sex allocation in subsequent generations, producing females that respond differently to the same environmental conditions, despite appearing otherwise similar.

Ethics

All experiments were performed under permits granted from the University of Tasmania Animal Ethics Committee (permit numbers A12366 & A13748).

Data Accessibility

This data has been stored at Dryad. doi:10.5061/dryad.b18gv

Competing Interests

We have no competing interests.

Authors' contributions

AE designed and coordinated the study, maintained the animals, carried out the experimental breeding procedures, carried out all dissections and molecular work, completed the statistical analysis and drafted the manuscript. EZC conceived the study, participated in the design of the study, assisted with statistical analysis, and helped draft the manuscript. JCP and MAF-S prepared the paint probes, assisted with the molecular work and helped draft the manuscript. EW participated in the design of the study and helped draft the manuscript. SRH and KT assisted with ideas, and undertook animal physical body measurements. All authors gave final approval for publication. Authors are listed in order of contribution.

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Chapter 3

Environmental mismatch: experimental evidence that maternal effects constrain sex allocation in the next generation

Edwards, A.M., Cameron, E.Z. and Wapstra, E. (2016). Environmental mismatch: experimental evidence that maternal effects constrain sex allocation in the next generation. *Behavioral Ecology*. Under Review.



Abstract

Maternal effects, the influence of the maternal phenotype on developing offspring, can cause lifelong physiological changes in offspring. These changes may be adaptive, pre-programming the offspring for certain environmental conditions, like high predation risk. However, when a mismatch occurs between the pre- and post-natal environments these effects may be detrimental. Sex allocation theories suggest that parents should differentially allocate to the sexes in relation to local environmental conditions and ability to invest, and the consequent fitness benefits of sons and daughters. However, if an individual experiences an environmental mismatch as a result of incorrect maternal programming, then the individual's ability to allocate to the sexes as predicted may be compromised by these physiological alterations. We created a mismatch between the environments experienced by pregnant laboratory mouse mothers and the later breeding environment of these gestated offspring by experimentally simulating a low stress environment during late gestation but not the postnatal environment. Once the female offspring reached maturity and bred, their sex ratios were significantly female-biased compared to their untreated counterparts. However, if the breeding female mice had matched environments, achieved by repeating the simulated low-stress environment during both her own gestational development and around the conception of her offspring, the sex ratio returned to parity. Without prior knowledge of gestational experience we would have incorrectly concluded that no adjustment occurred. Inconsistent results of previous empirical studies may be explained by physiological constraints on mothers, with mismatched pre- and post-natal environments masking some sex ratio adjustments.

Lay Summary

Your mother's experiences influence the sex of your babies. Female mice that are born to mothers treated with a stress-blocker while pregnant have more daughters than sons. However, if that female receives the same treatment as her mother at conception of her own litter, the sex ratio of her babies is 50:50. This is due to lifelong physiological changes in the female offspring as a result of the stress environment of her mother.

Introduction

Maternal effects are defined as the causal influences of the mother's phenotype or genotype on developing offspring (Mousseau and Fox, 1998), with profound effects on offspring life history through, for example, lifelong physiological changes in offspring (Maestripieri and Mateo, 2009; Marshall and Uller, 2007; Wolf and Wade, 2009). During gestation, the mammalian mother in particular has a prolonged period of contact during which the environment that the mother experiences can interact with the development of the offspring, particularly through the uterine environment, thereby affecting the offspring's phenotype (Maestripieri and Mateo, 2009). These maternal effects may be developmental, or the previous experiences of the parents may also be transmitted epigenetically (Lane et al., 2014). Furthermore, the uterine environment is also influenced by the offspring's siblings, similarly causing physiological changes (Ryan and Vandenbergh, 2002). Therefore, the uterine environment can have lifelong and extensive influences on the offspring through both maternal and sibling effects.

Pre-programming of offspring to environmental conditions through maternal effects can be advantageous, as it allows phenotypic plasticity of offspring to occur at a faster rate than would be seen by adaptation through natural selection (Champagne, 2008). For example, snowshoe hares (*Lepus americanus*) born during high predation years experience increased stress and exhibit decreased density of glucocorticoid receptors in their hippocampus as a result of prenatal glucocorticoid programming, lowering their susceptibility to stress, and allowing them to function under the stress of high predation (Sheriff et al., 2010). Conversely, those born in low predation years exhibited higher stress levels (Sheriff et al., 2010). However, environments are not static and therefore, the environment that the mother experiences during gestation may not be the same as the post-natal environment that the offspring experiences, this may result in decreased offspring fitness (reviewed in Uller et al., 2013). For example, high predation from lynx is one of the main causes of population crashes in the snowshoe hare which then remain low, despite the removal of the threat, due to intergenerational, maternally inherited stress hormones from the population decline period (Sheriff et al., 2010). Therefore, the mismatch between pre- and post-natal

environments can be detrimental to offspring (Kapoor et al., 2006). Artificially simulated increases or decreases in maternal stress during late gestation can result in a mismatched stress response in offspring, which may lead to abnormal predator responses (Kapoor et al., 2006), increased anxiety behaviours (Weaver et al., 2005) and decreased cognitive abilities (Hauser et al., 2009). Such alterations may then impact other life history traits, including survival and reproductive success (e.g. Sheriff et al., 2010).

Stress physiology has been linked mechanistically to sex allocation, both directly and through an interaction with glucose (e.g. Cameron et al., 2008; Moore et al., 2015; Ryan et al., 2012). Sex allocation hypotheses predict that parents should adjust the sex ratio of their offspring with local conditions or ability to invest, if fitness returns are sex-specific (Clark, 1978; Hamilton, 1967b; Silk, 1984; Trivers and Willard, 1973). For example, directional sex allocation is predicted where one sex is differentially advantaged in reproductive success by extra investment. Generally, studies support the trend (Cameron, 2004; Sheldon and West, 2004), but there remains a level of unexplained variation (West, 2009), and unpredictable effect sizes between individuals (Edwards and Cameron, 2014). This variation suggests the possibility of constraints imposed on a female's ability to respond to the environment (Edwards et al., 2016). Changes to baseline physiology as a result of maternal effects may explain some of the inter-individual variation (Edwards et al., in press).

Recently, we showed that an experimentally-induced low stress environment during late gestation caused physiological changes in the stress response of female offspring. This decreased the female's offspring sex ratio resulting in more daughters under normal environmental conditions, due to a mismatch between her pre- and post-natal environments (Edwards et al., in press). The same experimentally-induced low stress environment experienced only at the time of conception also decreased her offspring sex ratio (Cameron et al., 2008). Given that both treatments result in a female biased sex ratio in litters, we were able to test if sex biases are additive when both occur, or whether they are caused by mis-matched maternal effects, which would cause the bias to disappear if environments were matched. Here, we test the effects of these combined pre-natal and conception treatments on laboratory mice.

We test whether 1) the combined treatments result in an additive response of decreased offspring sex ratios, predicted if females are responding independently to each of the environmental treatments, or 2) whether the effects are negated due to pre- and post-natal environmental matching.

Methods

We used BALB/c mice bred and housed at the University of Tasmania, Australia. They were kept under 12hr L:D photoperiod in a temperature and humidity controlled room, and provided with mouse chow (Barastoc® irradiated food) and filtered water *ad libitum*. The control females utilised in this study are stock female mice from the colony; they have undergone no treatments.

Generating Focal Females

The experimental design is outlined in Figure 1. Twenty nulliparous dams were housed in groups of up to five until seven weeks of age when they were separated into pairs. One male was introduced to each cage, and remained with the females until mating was confirmed via presence of a copulatory plug.

We utilised the same females from Edwards et al. (in press), who had lowered stress during gestation as a result of dexamethasone treatment. Dexamethasone is a synthetic glucocorticoid that simulated an artificial low stress environment in the mothers (Cameron et al., 2008; Pratt and Lisk, 1990). Foetuses are very sensitive to glucocorticoids (Nyirenda and Welberg, 2001; Welberg et al., 2001), and therefore protective enzymes (e.g. 11 beta-hydroxysteroid dehydrogenase type 2) exists in the placenta to metabolise approximately 80% of naturally occurring glucocorticoids. Dexamethasone, however, is not metabolised by the placenta, and so the effects are expected to be exaggerated (Drake et al., 2005). The interaction of dexamethasone with the mother's body, and free dexamethasone interacting with the offspring results in a perceived low stress environment for the offspring.

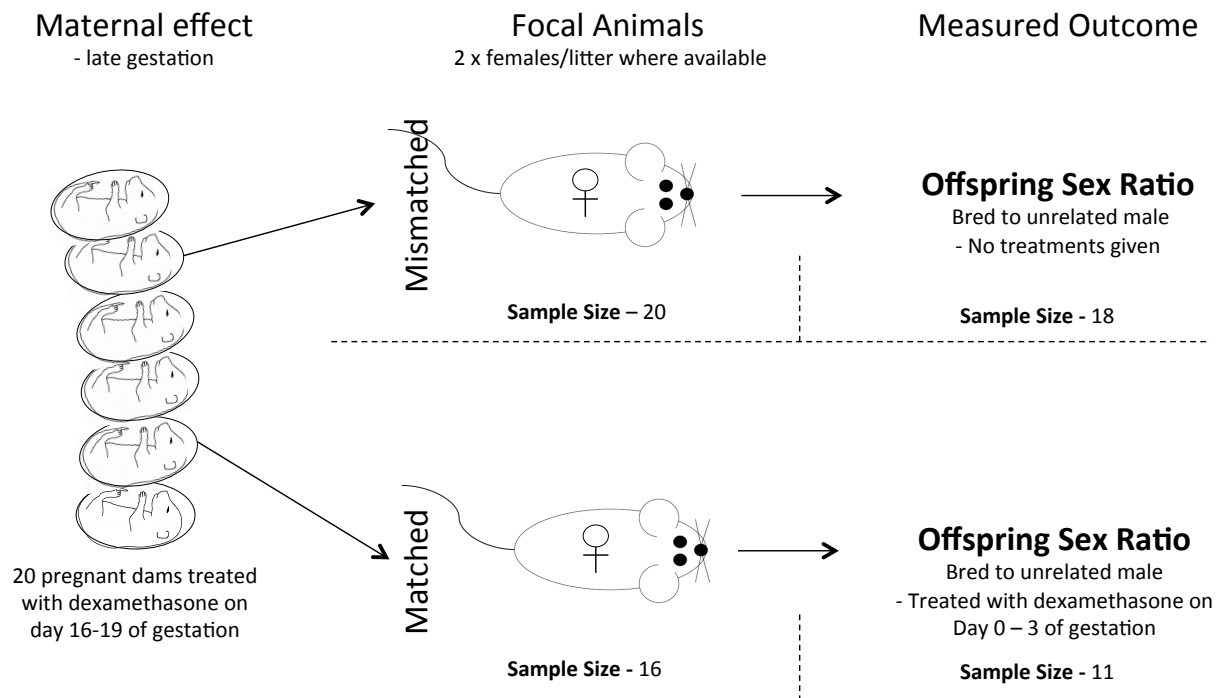


Figure 1. The experimental design of a sex allocation study investigating whether maternal effects influence a female's ability to respond to environmental pressure. The sample sizes at each stage of the experiment are listed.

At day 16 after the confirmation of a copulatory plug, $1.0 \mu\text{g ml}^{-1}$ of dexamethasone (as used by Cameron et al., 2008) was added to the drinking water of the dams, and this was replaced with fresh water after 3 days. Although this method results in variable dosages, it eliminates any increase in natural GCs from the stress of handling and injections (Pratt and Lisk, 1990), which potentially could negate the treatment (Cameron et al., 2008). The females were then left to litter without interruption. Two focal females from each litter were kept for the purpose of this study. Four dams only produced one female; these were used as mismatched females; resulting in 20 mismatched focal females, and 16 matched focal females. Immediately prior to mating, we calculated the body condition of the females from the residuals of an ordinary least squares linear regression of body mass and pes length (Schulte-Hostedde et al., 2005).

Breeding of Environmentally Mismatched Focal Females

Mismatched females were mated to unrelated males and allowed to birth naturally with pups being sexed by anogenital distance. One female did not conceive, and another committed infanticide prior to offspring sexing and so was removed from the analysis. The final sample size of environmentally mismatched females was 18 (Figure 1).

Breeding of Environmentally Matched Focal Females

On the day that the environmentally matched female was added to the male's cage for mating, the water was treated with $1.0 \mu\text{g ml}^{-1}$ of dexamethasone, this remained in the cage with the female until day 3 after the presence of a copulatory plug was noted. This treatment simulated a low stress environment, and therefore matched that of the prenatal environment. The females were then allowed to birth naturally and pups were again sexed using anogenital distance. Two females did not conceive, and three others committed infanticide prior to offspring sexing and so were removed from the analysis. The final sample size of environmentally matched females was 11 (Figure 2).

Statistics

We used generalised linear models (GLM) with binomial error and an intercept of 1 to verify whether the sex ratios of the two treatment groups and control laboratory mice differed from parity. We used an analysis of variance to determine whether the litter size between the matched and mismatched mice varied. We also used a generalised linear model with binomial error and a logit link to investigate the effects of environmental matching and body condition on offspring sex ratio.

All analyses were performed in R version 3.2.2 (R Core Team, 2015).

Results

The environmentally mismatched mice had sex ratios that were significantly lower than the expected 50:50 ratio (GLM: -0.839, -0.116; Figure 1), however neither the

environmentally matched group (GLM: -0.492, 0.492) nor the control mice (GLM: -0.657, 0.239) differed from parity. There was no difference in litter size between the matched and mismatched mice ($F_{1,27} = 2.46$, $\Pr(>Z) = 0.13$).

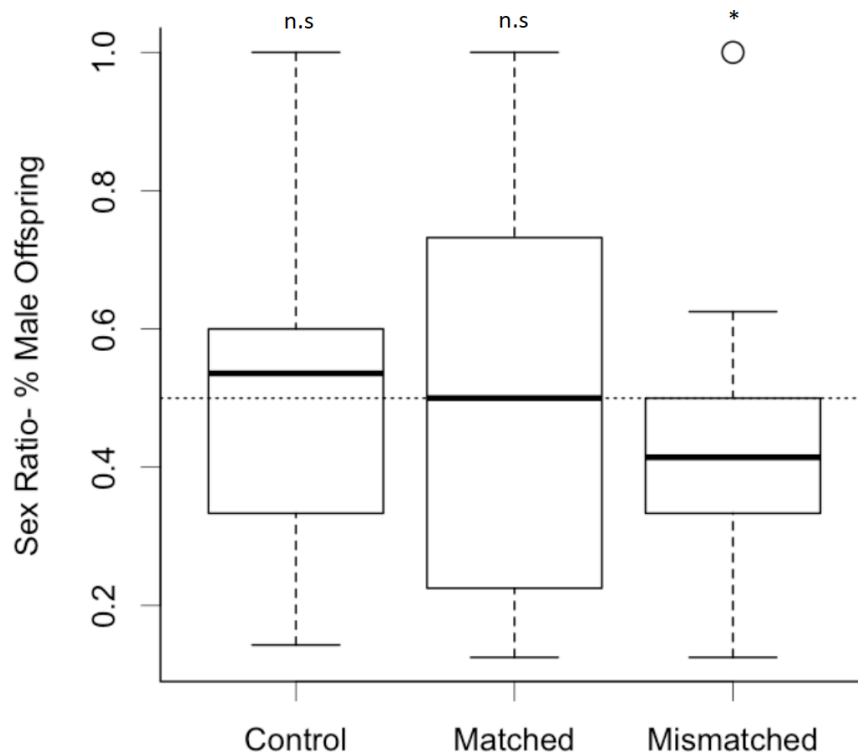


Figure 2. The offspring sex ratios from control laboratory mice, and those mice whose pre- and post-natal environments match are not significantly different from parity. While those mice whose pre- and post-natal environments don't match have sex ratios that are significantly lower than parity.

Note that '*' signifies a significant difference and 'n.s' signifies a non-significant difference from the expected 50:50 ratio. The dotted line indicates the expected 50:50 ratio.

The generalised linear model showed a trend towards the matched group having higher offspring sex ratios than the mismatched group ($\Pr(>\text{Chi})_{1,27} = 0.056$), but there was no indication that female body condition influenced sex ratios ($\Pr(>\text{Chi})_{1,26} = 0.301$).

Discussion

When the pre- and post-natal environments matched, females produced offspring sex ratios not different to the expected 50:50 ratio, as we see in control laboratory mice (which also experienced a unmanipulated, but matched, environment). This suggests that the artificially simulated environment at conception did match the environment for which they were pre-programmed. However, the mismatched mice, which were exposed to the maternal effect, but did not experience lowered stress at conception, produced offspring with a sex ratio lower than the expected 50:50 ratio, suggesting that the mismatch in environments led to physiological constraints on these females.

Due to the difference between the maternal environment and the conception environment in the mismatched mice, these mice produced sex ratios lower than the expected 50:50. Previously, we examined the differences between these mismatched mice and control mice (Edwards et al., in press), and suggested that lowered luteal cortisol (Dunn et al., 2010) and subsequently lowered levels of gluconeogenesis (Haynes and Lu, 1969) caused changes in free glucose levels (Drake et al., 2005) influencing offspring sex ratios (Cameron, 2004). This led us to question whether the significant change in sex allocation would remain if the maternal environment matched the peri-conceptual environment.

Lowered maternal stress levels during late gestation program the physiology of the offspring to be at its optimum in a matching environment (Kapoor et al., 2006). Therefore, using the same dosage of dexamethasone presented in the same manner at conception time, to lower the female's stress levels, should mirror the same environment that she was programmed for, and therefore, we would expect to see that the sex ratio of offspring remains at parity. Sex allocation theory suggests that parents should adjust sex ratios in relation to current local conditions or ability to invest (Clark, 1978; Hamilton, 1967a; Silk, 1984; Trivers and Willard, 1973), and here we see that the females are not differentially allocating under the treatment, as they perceived the environment to be neutral. Cameron *et al* (2008) used the same treatment at conception, on normal laboratory mice, and showed a significant decrease in their offspring sex ratios. An artificially lowered stress environment at

conception is perceived by normal laboratory mice to be an environmental condition under which differential allocation has been selected. However, due to the effect of the treatment in this study, these mice do not differentially allocate under the environmental pressure. They would, however, perceive the normal, standardised environment to be an environmental situation under which they should differentially allocate as seen in Edwards *et al.* (in press). Therefore, we reject the notion that the combined treatments has an additive effect and rather we support our hypothesis that mismatching maternal effects are capable of constraining a female's ability to respond to environmental conditions in the manner predicted by sex allocation theories (Edwards et al., 2016).

Sex allocation theories are based on the assumption that all mothers are equally capable of adjusting their sex ratios in relation to the current conditions, but variation in effect sizes in empirical studies suggests that this is not the case (Edwards et al., 2016). The ability of maternal effects to permanently adjust the physiological pathways involved in sex allocation suggests that we should take a broader look at a female's previous life experiences to fully understand her ability to appropriately allocate to the sexes. Specifically, our results suggest that developmental experience may alter female sex allocation, thereby, masking sex ratio skews, and potentially contributing to the variable effect sizes seen in previous studies. For example, without knowing the developmental history of our females that experienced a prenatal maternal effect, we would have predicted a female biased sex ratio with the conception treatment, and wrongly concluded that there was no sex ratio effect. Once we also considered the developmental impacts of our induced maternal effect, the constraints on maternal ability to alter sex ratios became apparent, and a sex allocation effect (return to parity) was shown. Previous studies have assumed that all mothers are similarly able to adjust the sex ratio in line with hypothetical predictions, but our study indicates that physiological constraints may violate this assumption and help to explain the inconsistent results of field studies of sex allocation. Mismatched maternal effects may therefore remove the adaptive benefits of maternal programming.

Improvements to livestock and conservation breeding programs (Robertson et al., 2006), as well as the prevention of sex related diseases in humans (Carvalho et al., 2012) requires further research into sex allocation and its physiological components; these results suggest that life history and experiences need to be accounted for in future studies. In particular, the profound influences of maternal effects on the development and future reproductive success of offspring and the interaction of these with current environmental conditions should be considered in studies of sex allocation and other life history traits. Further to this, future studies should expand to include K-selected species, whose life history differs remarkably to the rodents (R-selected) studied here. Due to the short, fast life of an R-selected species, the unstable and unpredictable environments may present little advantage to pre-programming, while the longer life of a K-selected species may uncover more benefits to maternal programming.

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Data Accessibility

This data will be made available on Dryad upon acceptance to Behavioural Ecology.

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Section II:

Paternal Influences



Chapter 4

Forgotten fathers: paternal influences on mammalian sex allocation

Edwards, A.M. and Cameron, E.Z. (2014). Forgotten fathers: paternal influences on mammalian sex allocation. *Trends in Ecology and Evolution*. 29 (3), 158-164.



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Edwards, A.M., Cameron, E.Z., (2014),
Forgotten fathers: paternal influences on
mammalian sex allocation, Trends in ecology
and evolution, 29(3), 49-57.

Chapter 5

Paternal sex allocation: how variable is the sperm sex ratio?

Edwards, A.M., Cameron, E.Z., Pereira, J.C. and Ferguson-Smith, M.A. (2016). Paternal sex allocation: how variable is the sperm sex ratio?. *Journal of Zoology*. **299**: 37-41.



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Edwards, A.M., Cameron, E.Z., Pereira, J.C.,
Ferguson-Smith, M.A. (2016). Paternal sex
allocation: how variable is the sperm sex
ratio?, *Journal of zoology*, 299, 37-41

Chapter 6

Cryptic male choice: experimental evidence of sperm sex ratio and seminal fluid adjustment in relation to coital rate.

Edwards, A.M. and Cameron, E.Z. (2016). Cryptic male choice: experimental evidence of sperm sex ratio and seminal fluid adjustment in relation to coital rate. *Reproduction, Fertility and Development*. In Press.



Cryptic male choice: experimental evidence of sperm sex ratio and seminal fluid adjustment in relation to coital rate

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Abstract. The differential allocation hypothesis suggests that a mother should adjust the sex of offspring in relation to her mate's attractiveness, thereby increasing future reproductive fitness when her sons inherit the attractive traits. More attractive males have been shown to sire more sons, but it is possible that the sex ratio skew could be a result of paternal rather than maternal manipulation, which would be a more parsimonious explanation. We manipulated coital rate (an indicator of attractiveness) in laboratory mice and showed that males that mate more often have higher levels of glucose in their semen despite lower blood glucose levels. Since peri-conceptual glucose levels *in utero* increase male conceptus survival, this could result in male-biased sex ratios. The males that mated most also had more remaining X-chromosome-bearing-spermatozoa, suggesting depletion of Y-chromosome-bearing-spermatozoa during mating. We hypothesise that males may alter both seminal fluids and X:Y ratios in an ejaculate to influence subsequent sex ratios. Our results further support a paternal role in sex allocation.

Additional keywords: attractiveness, differential allocation, paternal, sex allocation.

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Introduction

The differential allocation hypothesis of sex allocation (Burley 1986) suggests that females would benefit from producing sons when they mate with an attractive male, as sons would inherit those attractive qualities (e.g. Burley 1981). Attractiveness may be an honest indicator of male genetic (e.g. Andersson 1986) or sperm quality (Malo *et al.* 2005), and heritable traits that are passed onto sons will increase the overall fitness of the parents (sexy son hypothesis, Weatherhead and Robertson 1979). Therefore, it is beneficial for a mother to skew the sex ratio of her offspring in relation to her partner's attractiveness (Rutstein *et al.* 2005).

Previous studies have shown sex ratio biases in relation to paternal attractiveness (Røed *et al.* 2007). However, it is possible that such offspring sex ratio skews may arise paternally, as well as, or instead of, maternally. Sperm sex ratios seem more variable than previously thought (reviewed in Edwards and Cameron 2014), implying the possibility of adaptive paternal sex allocation (Edwards and Cameron 2014; Edwards *et al.* 2016a), and increasing evidence suggests that ejaculated sperm sex ratios are not equal (Saragusty *et al.* 2012; Edwards and Cameron 2014; Edwards *et al.* 2016a). The time delay between the initial stages of sperm production (i.e. meiosis, when ratios should be 50:50) and ejaculation provides an opportunity for the ratio to shift from parity. Random selective loss at various stages could explain some variation, but so might factors linked to investment strategies and attractiveness, such as male coital

rate (Edwards and Cameron 2014; Edwards *et al.* 2016a). Coital rate may indicate male attractiveness, particularly in those species with female choice, since males with more access to females may have more attractive traits and would therefore be advantaged by producing sons if they inherit these traits. Therefore, previous studies investigating maternal sex allocation in relation to paternal attractiveness may have incorrectly assigned variations to maternal adjustment in a situation where paternal explanations are more parsimonious (Edwards and Cameron 2014).

Rate of mating could influence sex allocation in different ways. The sexual dimorphism of the X and Y chromosomes could facilitate variation in sex ratios of spermatozoa in an ejaculate (Edwards and Cameron 2014). Fathers could also influence sex allocation through seminal constituents, rather than directly through sperm sex ratios (Edwards *et al.* 2016b). A complex assortment of components in seminal fluid serves many purposes (Perry *et al.* 2013) but the possibility that seminal fluids influence sex allocation has not been investigated, despite known impacts that some seminal fluid constituents (e.g. glucose) have on early conceptus survival and development (Larson *et al.* 2001; Cameron 2004). The production of sperm sex ratios may be relatively set within the male, due to sperm production times (Oakberg 1956), but the levels of seminal fluid constituents vary proximally between ejaculates (Perry *et al.* 2013).

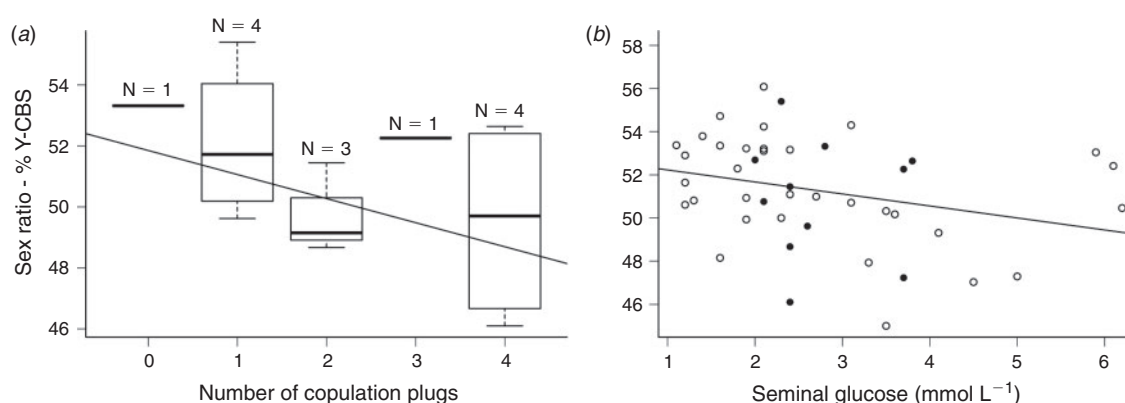


Fig. 1. The sperm sex ratio from male mice decreases as (a) the number of copulation plugs (confirmed matings) increases and as (b) the level of glucose (mmol L⁻¹) in the semen sample increases. Trend lines depict the linear relationships. (b) Open and closed circles indicate the virgin and mated males respectively.

Here, we experimentally test the relationship between coital rate and sperm sex ratios, as well as semen glucose levels, as an initial experimental indicator of the possibility of paternal control through seminal fluids in laboratory mice.

Methods

Ethics

All experiments were performed under permits granted from the University of Tasmania Animal Ethics Committee (permit numbers A12366 and A13748).

We used 15 male and 90 female BALB/c mice bred and housed at the University of Tasmania, Australia. Prior to experimentation the ano-genital distance and pes length of the male mice was measured using digital calipers. Body condition was calculated from the residuals of an ordinary least-squares linear regression of body mass and pes length (Schulte-Hostedde *et al.* 2005). Ano-genital distance was measured as an indicator of prenatal androgen exposure (Hurd *et al.* 2008).

Mating protocol

Female oestrus was brought on using the Whitten effect (Whitten 1966) and detected by swelling and redness around the vaginal area. A pair of oestrous, virgin females was assigned to each male, each night for 3 nights. The pair was introduced to the cage at 1600 hours on Night 1 and removed the following morning at 0800 hours, with the same procedure followed for the subsequent 2 nights, so that each male was allowed to access six unmated females over the 3 nights. Females were checked for the presence of a copulatory plug to determine successful mating. On the final morning of the experiment the males were sacrificed via cervical dislocation and prepared for dissection.

Sample collection

Blood glucose was measured immediately after death via tail tipping using an Accu-Chek Performa Nano (Roche). Spermatozoa for sex ratio analysis were collected and analysed as described in Edwards *et al.* (2016a). A semen sample was placed onto an Advantage II test strip (Roche) and analysed using an

Accu-Chek Advantage glucometer (Roche) in order to obtain an ejaculate glucose reading.

Statistical analysis

Two of the 15 samples broke during transit. The data from the remaining 13 individuals was combined with the sperm sex ratio data from the 34 virgin males presented in Edwards *et al.* (2016a).

A generalised linear model, with binomial error, was run on the full dataset to investigate whether the seminal glucose, virgin or mated status, blood glucose, body condition or ano-genital distance influenced the sperm sex ratios of the males.

The dataset was then reduced to only those males who had mated and again a generalised linear model, with binomial error, was run to investigate the same variables with the additional inclusion of the number of copulation plugs that were left by each male.

A linear model was run to determine whether blood glucose, body condition, ano-genital distance or mated status influenced the seminal glucose levels. The dataset was then subdivided into virgin and mated males. A linear model was run on the virgin males to determine whether blood glucose, body condition or ano-genital distance influenced their seminal glucose levels and a similar model was run on the mated males with the inclusion of the number of copulation plugs that were left by each male.

Results

The full statistical outputs can be found in Tables S1–S5, available as Supplementary Material to this paper.

From the data on mated males, those who had left more copulatory plugs experienced a decrease in the sperm sex ratio, indicating an increase in X-chromosome-bearing-spermatozoa (CBS; $\text{Pr}(>\text{Chi})_{1,8} = 0.00$; Fig. 1a; Table S2). The sperm sex ratio of the entire population was negatively correlated with their seminal glucose levels ($\text{Pr}(>\text{Chi})_{1,42} = 0.02$; Fig. 1b; Table S1), indicating a higher proportion of X-CBS as the glucose level increases. However, interestingly, there was no interaction between the virgin status and the seminal glucose level in regards to the sperm sex ratios.

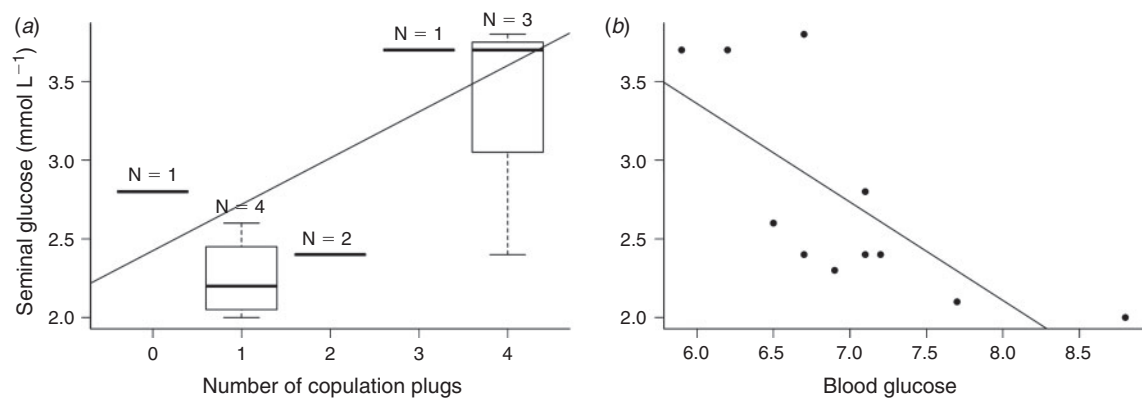


Fig. 2. (a) The seminal glucose level (mmol L⁻¹) in male laboratory mice increases as coital rate (indicated by number of copulation plugs) increases. (b) The negative trade-off between blood glucose (mmol L⁻¹) and seminal glucose (mmol L⁻¹) in male laboratory mice. Trend lines depict the linear relationships.

There was no indication that blood glucose, body condition, ano-genital distance or mated status influenced the level of seminal glucose in the entire population or within the virgin males (Table S1). However, an increase in seminal glucose in the mated males was seen when the number of copulation plugs left increased ($F = 11.73$; $\text{Pr}(> F) = 0.02$; Fig. 2a). There was also a negative relationship between the seminal glucose level and the blood glucose level ($F = 7.22$; $\text{Pr}(> F) = 0.04$; Fig. 2b).

Discussion

Males that mated the most had the highest semen glucose levels and more stored X-CBS. There was a negative relationship between blood glucose and semen glucose levels, indicating a potential trade-off in glucose allocation. If semen glucose levels alter the female reproductive tract, thereby altering sex-differential survivorship of early conceptuses, then glucose should be higher where sons would be advantageous to the father. Higher levels of peri-conceptual glucose decrease female conceptus survival (Larson *et al.* 2001; Cameron *et al.* 2008), due to toxic by-products resulting from X-linked metabolic pathways (Gutiérrez-Adán *et al.* 2001).

The males that mated the most had lower sperm sex ratios, opposite to our initial predictions. Mated males appear to have fewer spermatozoa; however, we were unable to quantify this. Previous studies have shown sperm counts decrease with successive ejaculations (Dewsbury and Sawrey 1984) and while there is contention regarding sex ratio skews in relation to ejaculation sequence (D'Amato *et al.* 1979), there is some evidence in support of this (e.g. Lloyd-Jones and Hays 1918). If this is the case, then mating frequency may result in biased Y-CBS depletion leaving more X-CBS stored in the epididymis. Males can adjust sperm number and semen quantity in response to female attractiveness and mated status (Cornwallis and O'Connor 2009) and so may skew ejaculates towards Y-CBS when the production of a son is beneficial, but this has not been tested. If the males produced Y-CBS-biased ejaculates during the experimental matings, the remaining semen would be depleted of Y-CBS, possibly explaining our X-CBS-biased samples. However, it is also possible that the sperm sex ratio

did not have time to adjust in the 3 days of the trial, due to sperm production times (Oakberg 1956) and so the sperm sex ratios may reflect the fact that the males were previously unmated.

The sperm sex ratio was negatively related to semen glucose level. We hypothesise that seminal fluids may counteract the X-bias in future ejaculates, thereby enhancing the probability of a son surviving to implantation. Adjustment of the seminal fluid substituents, such as glucose, may enhance the survivorship, swim speed or fertilisation rate of the spermatozoa or the survivorship of early conceptuses. We were not able to test this under the present experimental design, but our results suggest that further investigations are required. Sexual dimorphism exists between the X- and Y-CBS, in terms of both size (Carvalho *et al.* 2013) and swim speed (Check and Katsoff 1993), although some conclusions remain contentious (reviewed in Windsor *et al.* 1993). Glucose increases beat frequency in spermatozoa (Mannowetz *et al.* 2012) and so they may utilise this within the uterine environment to reach the fertilisation site. However, seminal fluids may be adjusted by the male to use these differences between the X- and Y-CBS to increase sexually dimorphic fertilisation or early conceptus survival.

The ability to influence offspring sex has generated a significant amount of interest, particularly due to the possible improvements to breeding programs, livestock industry and human applications in the prevention of sex-related diseases (Carvalho *et al.* 2013). Further research is required to investigate the changes in sperm sex ratios across time and with paternal conditions, such as coital rate. We also suggest that future research consider the possibility that seminal fluids could influence sex allocation.

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Chapter 7

General Discussion



Despite the logical simplicity of the theoretical basis of sex allocation, empirical studies have typically produced inconsistent results (West, 2009). Therefore, support for adaptive sex allocation hypotheses has been contentious (Cameron, 2004; Sheldon and West, 2004) but recent studies have attributed much of the variation in results to methodological inconsistencies (Cameron, 2004). However, even when accounting for methodological variation, the vast majority of variation in effect sizes, both between and within species remains unexplained (West, 2009). This unexplained variation forms the basis of my thesis. Initially, I explored potential sources of variability in effect size by considering potential constraints on maternal ability to adjust sex ratios and I was the first to suggest the possibility of physiological constraints impacting maternal sex allocation in mammals (Chapter 4; Edwards and Cameron, 2014; Chapter 1; Edwards et al., 2016b). Three physiological constraints were identified as likely to be impacting females during sex allocation; these were life-long and inter-generational modifiers of maternal physiology, proximal modifiers of maternal physiology, and constraints imposed by fathers. My thesis then consisted of testing the potential constraints that I identified. Section I (Chapters 2-3) of this thesis investigated whether lifelong physiological changes as a result of maternal effects are capable of constraining a female's ability to appropriately allocate to the sexes. Section II (Chapters 4-7) investigated the possibility that fathers may be influencing maternal sex allocation through sperm sex ratio skews and seminal fluid proteins.

In section I, I investigated whether maternal effects, the causal influence of the maternal phenotype or genotype on her developing offspring (Mousseau and Fox, 1998), were capable of constraining a female's ability to respond to her local conditions, and subsequently differentially allocate to the sexes as predicted once she reaches maturity (Chapter 2; Edwards et al., in press). Down-regulated stress during late gestation did not cause physical body changes to the offspring, however as was evident through sex ratio skews in the next generation, the physiological pathways were adjusted in line with the maternal effect. Females who experienced down-regulated stress during their development decreased the sex ratio of their offspring, however differing levels of blood glucose at the time of conception explained more of the variation in sex ratio skews than did the treatment itself. We hypothesized that

the skewed sex ratios produced by these females were due to the mismatch between pre- and post-natal environments, as determined by the artificially lowered stress maternal effect.

In order to test whether the mismatch in pre- and post-natal environments was responsible for the sex ratio skew, I took another group of females who were exposed to the same maternal effect, and artificially lowered their stress levels at conception, to match the environment that they were pre-programmed for (Chapter 3). These females produced sex ratios that were not different to the expected 50:50 ratio, and were not different to that of normal untreated laboratory mice. Mice that have not received a maternal effect treatment but that are exposed to artificially lowered stress at conception will decrease their sex ratios (Cameron et al., 2008), as they interpret the environment to be one in which they should differentially allocate. However, maternally effected mice do not react to the environment in the same manner, as their physiology had been adjusted to suit that which the mother experienced during late gestation. These results support my hypothesis that lifelong and intergenerational modifiers of maternal effects are capable of constraining a female's ability to respond to the environment when the pre- and post-natal environments do not match.

In section II of my thesis, I investigated whether paternal influences constrain maternal sex allocation, through both sperm sex ratios and seminal fluids. Previously, both theoretical hypotheses and empirical studies have assumed that the paternal contribution to sex allocation is absent, due to meiosis controlling the sperm sex ratio at the initial stage of production. I established, through a review of the literature, that sperm sex ratio biases are present at the population and individual level, and surprisingly even between ejaculates from the same male (Chapter 4; Edwards and Cameron, 2014). Skews in sperm sex ratios exist under many circumstances, with exposure to environmental contamination, fertility, sexual rest, age and diet among some of the possible explanations for the skews.

However, environmental contamination, fertility, age and diet may present data on sperm sex ratios under non-normal conditions, so I investigated whether sperm sex ratio skews existed under normal, standardised conditions in a model species, the

laboratory mouse (Chapter 5; Edwards et al., 2016a). Interestingly, not only was there variation at the individual level, this variation was so large that the population average did not mask this effect. Overall, there was a propensity to have increased Y chromosome-bearing-sperm (CBS) in virgin laboratory mice.

Further to this, I then tested experimentally whether sperm sex ratios and seminal fluids were adjusted with mating rate (Chapter 6; Edwards and Cameron, 2016). Mating rate may be an indicator of male attractiveness, which has previously been associated with birth sex ratio skews (Roed et al., 2007). The differential allocation hypothesis (Burley, 1986) states that females should invest in sons when the father is attractive (Burley, 1981), as heritable traits that are passed onto sons will increase the overall fitness of the parents (Weatherhead and Robertson, 1979). However, here I presented evidence that males adjust both sperm sex ratios and seminal glucose in line with the prediction of an increase in male offspring, as is expected under the differential allocation hypothesis that increased male attractiveness should result in increased sons. This provides initial evidence to support the theory that paternal experiences may drive sperm sex ratios and seminal fluids, and that these components may constrain maternal sex allocation.

In this thesis, I have tested different types of constraints on a mother's ability to adjust the sex ratio of her offspring in accordance with their local conditions and ability to invest. This finding requires that future sex allocation expand beyond that of the current confines of the field to further include previous life and developmental experiences of females, their infection and disease status, as well as the life experiences of the father. Importantly, evidence suggesting that the paternal contribution to sex allocation is not absent which may require reconsideration of previous empirical studies, as this may explain why some results have previously not conformed to theoretical expectations.

The implications of my results extend beyond sex allocation research. For example, studies that utilise some genetic markers assume equal input from fathers into the population, which now may require reconsideration. Studies using Y-chromosome microsatellite markers and diversity (e.g. Eriksson et al., 2006; MacDonald et al.,

2014) assume gene flow is not influenced by sex ratios. Similarly, many population processes in ecology work under the assumption the father's contribute equal numbers of X- and Y-CBS. Now that we are aware of the extent of variation within and between individuals' sperm sex ratios we can begin to reassess these processes.

The ability to influence offspring sex has generated a significant amount of interest for a long time, not only as a fundamental question in evolutionary biology and population dynamics, but also due to the improvements that could be made to livestock and breeding programs, as well as human applications in the prevention of sex related diseases (Carvalho et al., 2012). However, as it currently stands, sex allocation research is taking a limited approach to investigating what drives large sex ratio biases, and my research brings the conclusions of those previous studies into contention as it questions the validity of their assumptions and results.

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Supplementary Material



Supplementary Material

Cryptic male choice: experimental evidence of sperm sex ratio and seminal fluid adjustment in relation to coital rate

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Supplementary Material 1

Statistical Outputs: The results from the statistical models run on sperm sex ratios and seminal glucose levels in laboratory mice.

Table S1. The output of a generalized linear model, with binomial (logit link) error, investigating influences on the sperm sex ratio of male laboratory mice

Significant factors highlighted in bold

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
NULL			43	65.86	
Sperm Glucose	1	5.89	42	59.97	0.02
Virgin Status	1	0.15	41	59.82	0.70
Blood Glucose	1	0.72	40	59.10	0.40
Body Condition	1	0.29	39	58.82	0.59
Ano-genital Distance	1	0.04	38	58.78	0.85

Table S2. The output of a generalized linear model, with binomial (logit link) error, investigating influences on the sperm sex ratio of mated male laboratory mice

Significant factors highlighted in bold

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
NULL			10	19.64	
Sperm Glucose	1	0.03	9	19.61	0.86
Copulation Plugs	1	8.99	8	10.62	0.00
Blood Glucose	1	0.55	7	10.06	0.46
Body Condition	1	0.08	6	9.98	0.77
Ano-genital Distance	1	0.97	5	9.01	0.33

Table S3. The output of a linear model investigating influences on the seminal glucose level (mmol/L) of mated male laboratory mice

Including an interaction factor between the number of copulation plugs left and blood glucose level. Significant factors highlighted in bold

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Copulation Plugs	1	1.81	1.81	11.73	0.02
Blood Glucose	1	1.11	1.11	7.22	0.04
Body Condition	1	0.01	0.01	0.04	0.85
Ano-genital Distance	1	0.18	0.18	1.16	0.33
Copulation Plugs:					
Blood Glucose	1	0.60	0.60	3.92	0.10
Residuals	5	0.77	0.15		

Table S4. The output of a linear model investigating influences on the seminal glucose level (mmol/L) in male laboratory mice

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Blood Glucose	1	2.89	2.89	1.70	0.20
Body Condition	1	1.77	1.77	1.04	0.31
Ano-genital Distance	1	0.21	0.21	0.12	0.73
Mated Status	1	1.55	1.55	0.91	0.35
Residuals	39	66.47	1.70		

Table S5. The output of a linear model investigating influences on the seminal glucose level (mmol/L) of virgin male laboratory mice

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Blood Glucose	1	2.05	2.05	0.95	0.34
Body Condition	1	3.52	3.52	1.63	0.21
Ano-genital Distance	1	0.10	0.10	0.04	0.84
Residuals	29	62.73	2.16		